Microiontophoresis and Pressure Ejection

Peter M. Lalley

Introduction

The ability to introduce minute amounts of drugs and chemicals by iontophoresis or pressure ejection from fine-tipped micropipettes into the microenvironment of nerve cells offers several advantages over other modes of administration: (1) Direct application circumvents diffusional barriers and limits enzymatic breakdown that might otherwise prevent substances from reaching the site of action. (2) The actions and effects of the substances can be confined to a single neuron. (3) Cell receptors for neurotransmitters and neuromodulators can be identified pharmacologically. (4) The functional significance of neurotransmitters can be assessed by comparing their effects through local application with stimulus-evoked responses, and observing how each are affected by receptor antagonists or by agents which block neurotransmitter breakdown or uptake. (5) Local application of membrane-permeable second messenger-selective agents can be used to identify intracellular signal transduction mechanisms.

The iontophoretic technique for applying neuroactive substances directly to neurons in the CNS was first utilized over 60 years ago. In 1936, Suh et al. identified cholinoceptive pressor regions in the brain stem by iontophoresing acetylcholine intracisternally. Later, Nastuk (1953) and Del Castillo and Katz (1955, 1957a,b) reintroduced the method to investigate the actions of acetylcholine at the neuromuscular junction. Curtis and Eccles (1958a,b) utilized multibarrel microiontophoresis to study the actions of pharmacological agents on spinal Renshaw cells. Since then, the technique has been used in numerous investigations to study the actions of various neurotransmitter candidates and neuroactive chemicals at synapses throughout the CNS (Krnjevic´ and Phillis 1963; Curtis 1964; Krnjevic 1964; McLennan and York 1967; Phillis et al. 1967; Salmoiraghi and Stefanis 1967; Curtis and Crawford 1969; Bradley and Candy 1970; Diamond et al. 1973; Hill and Simmonds 1973; Krnjevic 1974; Bloom 1974; Simmonds 1974; Lalley 1994; Lalley et al. 1994, 1995, 1997; Parker and Newland 1995; Young et al. 1995; Bond and Lodge 1995; Wang et al. 1995; Haji et al. 1996; Schmid et al. 1996; Heppenstall and Fleetwood-Walker 1997; Remmers et al. 1997; Zhang and Mifflin 1997). The technique of pressure-injecting substances from micropipettes was introduced by Reyniers in 1933 and improved by Chambers and Kopac (1950; see Keynes 1964). Krnjevic and Phillis (1963) applied glutamate by pressure-ejection onto single cerebral cortical neurons. Later, McCaman et al (1977), Sakai et al. (1979) and Palmer et al. (1980) published detailed accounts for micropressure-injecting known volumes of substances intracellularly and extracelluarly in the CNS. The method has proven to be very effective in measuring the actions and effects of poorly-charged substances on neurons (Dufy et al. 1979; Siggins and French 1979; Palmer and Hoffer 1980; Palmer et al. 1980; Sorensesn et al.

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1981; Palmer 1980, 1982; Palmer et al 1986). Intracellular pressure-injection has been used to stain neurons (Sakai et al. 1978), to demonstrate regulation of acetylcholine content by acetylcholinesterase (Tauc et al. 1974), track the transmembrane flux of calcium with fluorescent dyes (Chang et al. 1974), and study neurotransmitter synthesis and axonal transport (Schwartz 1974; Thompson et al. 1976). The technique has been utilized in awake animals for extracellular (Suvorov et al. 1996) and intracellular (Szente et al. 1990) application of neurochemicals.

In this chapter, methods for applying substances by microiontophoresis or micropressure-ejection to neurons in the CNS will be described. Emphasis will be given to methods used in the *in vivo* preparation. The techniques have also been used to study drug actions in brain and spinal cord slices and other types of *in vitro* preparation (Andrade and Nicoll 1987; Nicoll et al. 1990).

The reader is encouraged to read the more detailed discussions of microiontophoresis and micropressure-ejection in Curtis (1964), Bloom (1974), Simmonds (1974), Hicks (1984) and Palmer et al. (1986).

Subprotocol 1
Microiontophoresis

General Description

Microiontophoresis involves the controlled ejection of charged substances from glass micropipettes into the extracellular milieu of nerve cells or into a cell's cytoplasm. Between ejections, the substance is retained by applying current opposite in polarity to the ejecting current. For intracellular microiontophoresis, a single micropipette can be used to record membrane potential and inject substances with current in bridge mode (Chapter 5). Alternatively, theta glass tubing (which has a dividing septum) may be used. One side of the divided micropipette is filled with a solution of the chemical to be ejected and is fitted with a silver wire connected to a current pump, the other side contains the recording solution and is connected to the recording amplifier.

For extracellular microiontophoresis, it is common to eject several test substances near a neuron from a multibarrel array. A number of glass tubes are fused together and pulled to give an array of micropipettes, each containing a solution of a neuroactive substance. The tip of the array is bumped with a glass rod under microscopic control, such that each barrel has a final diameter of about 1 µm. One of the barrels is typically filled with 2–4 M NaCl for extracellular recording. Figure 1A illustrates a 5-barrel configuration. The center barrel would be used for recording while the other 4 barrels would be used to eject neuroactive chemicals. The parallel microelectrode array which is seen in Figure 1B was designed by Curtis (1968), modified by Oliver (1971) and is used in experiments involving intracellular recording and extracellular microelectrophoresis. A micropipette for intracellular recording is glued to a multibarrel pipette assembly for microiontophoresis. The arrangement allows one to monitor the effects of neuroactive chemicals on membrane potential and membrane currents. Substances can also be injected into target neurons to identify transduction mechanisms that couple membrane receptor activation to excitability (Lalley et al, 1997; Richter et al, 1997). The array in Figure 1C is a photomicrograph of a coaxial assembly from Remmers et al (1997) that was designed by Sonnhof (1973) for intracellular recording and extracellular microiontophoresis

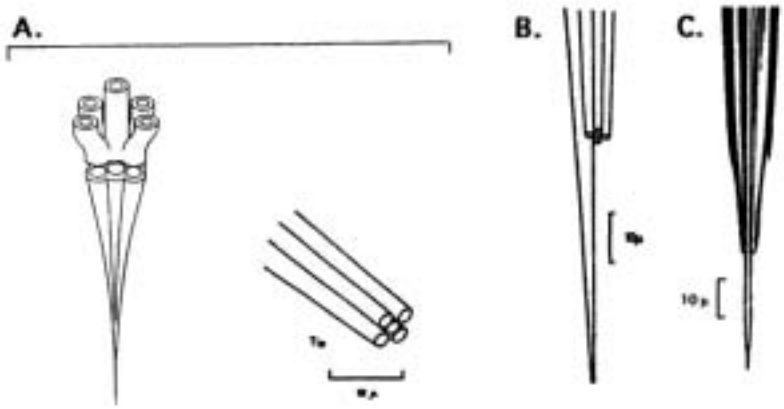

Fig. 1. Multibarrel arrays for microiontophoresis and recording from nerve cells. **A**: Drawing of five-barrel array for extracellular recording and local application of neurochemicals. *Left*, overall configuration. *Right*, tips at high magnification. Center barrel is typically filled with 2–4 M NaCl solution and used for recording. (Modified from McLennan 1970). **B**: Drawing of parallel micropipette assembly for intracellular recording and extracellular chemical application. Five-barrel array is glued in parallel to intracellular recording pipette. **C**: Photomicrograph of coaxial array for intracellular recording and extracellular microiontophoresis. A long, slender micropipette for intracellular recording is inserted down the center barrel of a multibarrel pipette assembly. Surrounding barrels are used for iontophoresis. (Modified from Remmers et al. 1997)

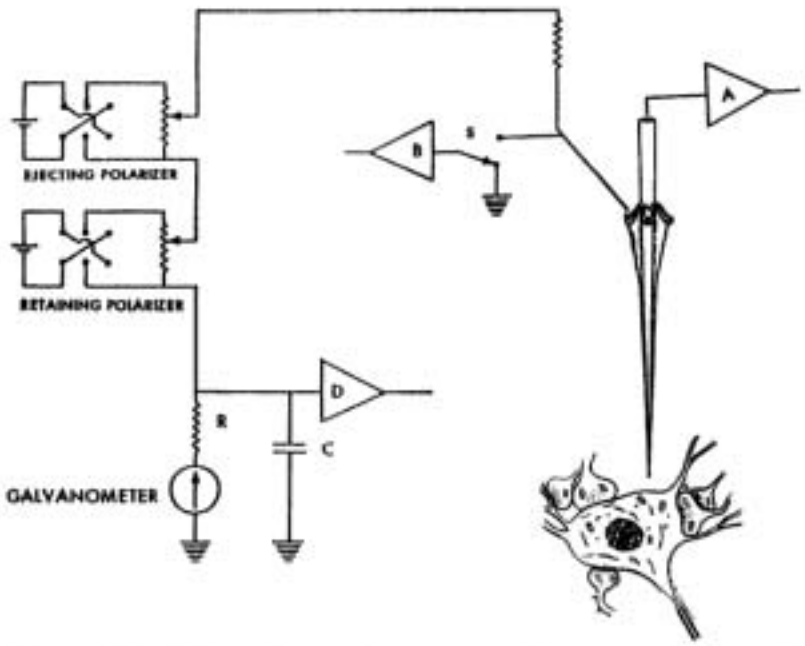

Fig. 2. Simplified electrical circuit for microiontophoresis. Amplifiers D and B and the galvanometer are used to monitor ejecting and retaining currents drawn from the polarizers. Amplifier A monitors bioelectric potentials. (Modified from Curtis 1964).

A simplified electrical circuit for microiontophoresis and extracellular recording is illustrated in Figure 2. See Curtis (1964) for a detailed explanation. One of the barrels is shown connected to ejecting and retaining "polarizers", which in former times were 90V and 45V batteries. More sophisticated constant-current pumps are now used to eject and retain substances and to measure the respective currents.

Materials

Basic Equipment Needs

The essential equipment items for iontophoresis experiments include:
- Equipment required for extra- or intracellular recording (see Chapter 5)
- Vertical pipette puller capable of generating sufficient current and pull for preparation of multibarrel micropipettes
- Heavy-duty heating coils for use with the pipette puller
- Programmable current pump for microiontophoresing neuroactive substances
- Window discriminator-ratemeter. Action potentials are detected in a voltage window. Each spike within the window triggers a TTL pulse which is passed to a ratemeter and to other counting and processing devices
- Computer interface, microprocessor, and software programs for constructing histograms and for various types of data analysis
- Pulse generator and stimulus isolation unit for evoking action potentials in response to stimulation of peripheral sensory or motor nerves or nerve tracts.

Choosing a Microiontophoresis Current Pump

Circuits for constant-current microiontophoresis can be constructed from published designs (for example, Walker et al, 1995). Alternatively, excellent multichannel current generators are commercially available (see equipment list at the end of this chapter). The microiontophoresis pump should have the following features:
- Several independently programmable channels to control ejecting and retaining currents
- Low noise properties
- Numerical display of currents
- Timers that switch on and switch off currents, so that substances can be pulsed or applied continuously
- Inputs and circuits that enable computer-control of currents
- Event pulses to chart recorders, oscilloscopes and computers signaling the occurrence and magnitude of retaining and ejecting currents
- A monitor for each channel to signal blocking of micropipette currents
- Capability to measure micropipette resistance
- A grounding switch for each channel
- Small headstage that can be positioned close to the preparation. Headstage should be equipped with a grounding post.

Procedure

Procedure 1: Preparation of Microelectrode Assemblies

Glass capillary tubing containing filaments or septa are used to facilitate filling of micropipettes. Tubing can be purchased already fused into 2–7 barrel arrays. Their advantage is that they need only be cut at appropriate lengths and pulled with a vertical pipette puller to the desired length and configuration before filling with drug solutions. The disadvantage is that care must be taken in filling to avoid spillover during filling from one barrel to another.

The alternative is to fabricate multibarrel arrays from single tubing and bend the tubes away from each other at the filling ends. The method described here is a modified version of the method described by Crossman et al. (1974).

Protocol 1

1. Capillary tubes are bundled together and held securely in place about 15 mm from each end with cuffs made from heat-shrinkable tubing.
2. The array is fixed firmly in the chucks of a vertical pipette puller. Heat is applied through the coil, with the magnetic pull set to zero, until the array is just soft enough to begin lengthening under the influence of gravity. Manual pressure is applied to allow only a small amount of lengthening, the array is rotated quickly through 270 degrees and the heating current is switched off to allow the glass to cool. The position of the coil is adjusted to the mid-region of the twisted, tapered glass column. Heating current and magnet pull are adjusted to desired levels and the array is pulled, yielding two usable multibarrel pipettes.
3. A metal hook is inserted into the open end of a barrel while applying heat to the shank from a Bunsen burner in order to bend the barrel by about 30 degrees. All barrels are bent in this fashion except the recording barrel.
4. Melted dental wax is applied between the bent and unbent barrels to form a crown. The crown and the cuff of heat-shrinkable tubing keep the barrels securely together. The micropipette array is then ready for filling and bumping to the proper tip diameter.
5. The recording barrel is connected to a manipulator and microdrive that advances the assembly into tissue.

Procedures for fabricating parallel and coaxial multibarrel arrays for intracellular recording and microiontophoresis have been described in detail (Curtis 1968; Oliver 1971; Sonnhof 1973; Lalley et al. 1994; Remmers 1997). The parallel electrode is simpler to fabricate and coupling resistances and capacitances are lower. The recording pipette must be strongly bonded to the electrophoresis assembly or they will separate in tissue. The coaxial type of electrode is much more difficult to fabricate; however, the recording and iontophoresing pipettes cannot separate.

The parallel array utilized by Lalley et al. (1994) is produced according to the following procedures and specifications:

Protocol 2

1. The distance between the recording tip and those of the microiontophoresis assembly is kept at 40 µm.
2. Single micropipettes for intracellular recording are pulled on a horizontal puller to yield the desired length, profile and tip. The recording barrel is bent with heat applied from a heating coil at an angle of 15–20 degrees, 10 mm from the tip.
3. The shaft of the recording barrel below the bend is positioned under microscopic control (400x amplification) in the crease between two barrels of the multi-barrel iontophoresis assembly.
4. Light-sensitive dental cement (3M Corporation, USA) is applied to the crease before bringing the two components together.
5. After correct positioning, the connection is made secure by applying 900 nm light from a curing gun for 30 seconds, then a second layer is applied to enclose the barrels and light-cured.

6. Cranioplast cement is applied at the bend in the recording barrel to make a small collar that further prevents separation.

The final product produces no more displacement of tissue than a standard 5-barrel array for extracellular microiontophoresis. This makes it highly suitable for recording responses of neurons deep in tissue (Richter et al. 1996).

Parallel array microelectrodes have been utilized for extracellular recording and microiontophoresis by Crossman et al. (1974). The tip of the recording pipette protrudes beyond the multibarrel assembly by 5–15 µm. The assemblies have excellent recording properties, with large signal-to-noise ratios, and are reported to be quite useful for recording responses of small cells in subcortical regions.

Carbon Fiber Electrodes

Low-noise recording electrodes for extracellular recording and microiontophoresis have been constructed by placing a carbon filament in the recording barrel of a multibarrel array and etching the fiber to produce a fine tip that is very suitable for single unit recording (Armstrong-James and Millar 1979; Fu and Lorden 1996). Multibarrel micropipettes with carbon fibers in two barrels have been used to record neuron discharges and to measure concentrations of iontophoresed catecholamines and 5-hydroxytryptamine (5-HT) (Armstrong-James et al. 1981; Crepsi et al. 1984).

Preparing Solutions

Procedures and precautions used in filling micropipettes for microiontophoresis are similar to those described for preparation of recording micropipettes in Chapter 5. The solutions should be filtered through 0.2 µm filter membranes to remove debris. The tips must be free of bubbles, otherwise the micropipettes will fail to iontophorese adequately and current passed through the filling solution will generate noise which will be picked up by the recording pipette. It is advisable to fill the micropipettes at least 10–15 minutes before use and store them in vertical position in a humidified chamber. The barrels should be checked microscopically for bubbles and precipitate immediately before use. Bubbles can sometimes be displaced by twirling a clean cat whisker in the barrels.

Solutions should be adjusted to a pH that will facilitate ejection of charged substances by iontophoresis or electro-osmosis without altering the substance's pharmacological properties. In solutions of pH 4 or less the neuronal actions of monoamines – which can excite or depress neurons depending on the subtype of catecholamine- or 5-HT receptor activated – are biased in favor of excitation (Frederickson et al. 1971). Responses related to pH are generally avoided when the pH of solutions are kept between 4.5 and 8.

Electrical Coupling to the Current Source

Clean silver wire should be used to apply current to the pipette solution. Chloriding is not necessary, and, in fact, may cause blockage due to flaking off of silver chloride, which clogs micropipette tips. To prevent contact and transfer of current between silver wires, it is convenient to solder a 60–70 mm length of silver wire to an 80 mm length of thin, flexible insulated cable and connect the latter to the iontophoresis current pump. The insulated cable can be fitted to an amphenol pin for connection to the headstage of the current pump. After the wires for iontophoresis and recording are inserted into the micropipette barrels, they are separated and held securely in place by carefully pouring a small amount of melted dental wax or paraffin over the pipette orifice.

Micropipette Tip Size

Micropipettes fill more readily when they are filled before bumping; however, they can be successfully filled even after bumping. Bumping is performed under microscopic control, with a glass rod mounted on a micromanipulator, to yield individual tip diameters of approximately 1 µm. This tip size generally results in low tip potentials. Larger tip diameters increase the likelihood of uncontrolled diffusion of substances.

Procedure 2: Recording and Microiontophoresis

1. Neurons are searched for using procedures described in Chapter 5.
2. The current pump is turned on when the micropipette tips are in the tissue. Normally, retaining currents of 5–10 nA are used. Ejecting currents are set to relatively low levels, such as 5 nA, and increased gradually to 60–80 nA. This procedure is used to test the current-passing properties of each micropipette. If blocking or unacceptable noise occurs, the assembly should be withdrawn and discarded.
3. It is advisable to first evaluate the effectiveness of test substances and ejecting currents on cells other than the target neurons. Such tests may be impractical for intracellular recording and extracellular microiontophoresis since damage to the recording tip might occur, but they should be performed whenever possible. Any unidentified neuron that fires spontaneously without an injury discharge can be tested for this purpose.
4. Microelectrophoresis pipettes need to be warmed up by passage of ejecting current. Sometimes, passage of even large ejecting currents will initially elicit no response since retaining currents have evacuated the test substance from the solution near the tip of the micropipette. This dead space will have to be recharged by repeated passage of ejecting current. A useful procedure is to apply ejecting currents of increasing intensity at fixed intervals with a 50% duty cycle, up to 100 nA. Once a response is elicited, tests should be repeated several times to insure that stable responses are evoked, indicating that the concentration of substance has reached equilibrium at the tip of the micropipette.
5. The multibarrel assembly should now be ready for tests on the intended neurons. Target neurons should be selected which have stable recording properties. Tests should not be made on neurons which exhibit injury discharges or action potential inactivation. In tests made with extracellular recording, action potentials should be of negative polarity and be at least several hundred μV greater than background noise. It is highly desirable to record the neuron in isolation from other neurons. If this is not possible, window discrimination may be used to count the spike frequency of the test neuron. In that case, it is important that the test substances are affecting only the target neuron. Cells tested during intracellular recording should exhibit stable membrane potential properties as described in Chapter 5.
6. Precautions to prevent current artifacts should be taken. Currents can evoke responses independent of the substance ejected, especially when the microiontophoresis assembly is close to a neuron. Current artifacts should be suspected if action potential frequency or membrane potential suddenly changes, coincident with the time course of the applied current. Generally, positive current inhibits discharges and hyperpolarizes membrane potential whereas negative current has opposite effects. During extracellular recording, current artifacts rarely occur if action potentials are negative in polarity, indicating that the assembly is sufficiently distant to prevent current spread to the cell membrane. A procedure to minimize current artifacts involves current-balancing. One micropipette of the assembly is filled with 165 mM NaCl and connected by silver wire to the balancing channel of the microelectrophoresis unit. The channel delivers current equal and opposite in polarity to the sum of currents delivered through all other barrels. A second procedure is to test for current artifacts by passing current through a barrel containing only 165 mM NaCl.
7. The adequacy of retaining currents should be determined. Normally, 5–10 nA currents will be adequate; however, the effectiveness of retaining currents should be questioned if responses to neuroactive substances are unexpectedly persistent or if responses change with a gradual time course when the intensity of the retaining current is varied. Currents greater than 20 nA are generally avoided since they produce dead space at the micropipette tips.

Overview 1: Measuring and Interpreting Responses

Spike Frequency Analysis

Action potential frequency can be measured as either instantaneous frequency, or as the moving average after processing by a leaky integrator circuit (Eldridge 1971). Moving averages can provide information about how neuroactive substances affect the pattern as well as the frequency of cell discharge. Another approach is to collect the spikes into time bins by computer. Histograms can be constructed which illustrate spike frequency or numbers of spikes vs. time. In addition, interspike interval histograms (ISIHs) or post-stimulus histograms (PSTHs) can be constructed. ISIHs can reveal whether neurochemicals preferentially depress either high- or low-frequency components in a train of action potentials (Hoffer et al. 1971). Figure 3 illustrates the use of moving averages and ISHSs to measure the depression of spike frequency in medullary inspiratory neurons by DAMGO, a μ-opioid receptor agonist (Lalley et al. 1997).

PSTHs are useful for analyzing drug effects on stimulus-evoked monosynaptic spike discharges (Bloom 1974).

Membrane Potential and Neuron Input Resistance

Intracellular recording reveals how chemicals affect subthreshold events such as resting membrane potential and membrane current, PSPs and PSCs, membrane conductances and input resistance, R_N, and discharge properties. Measurements of R_N will often indi-

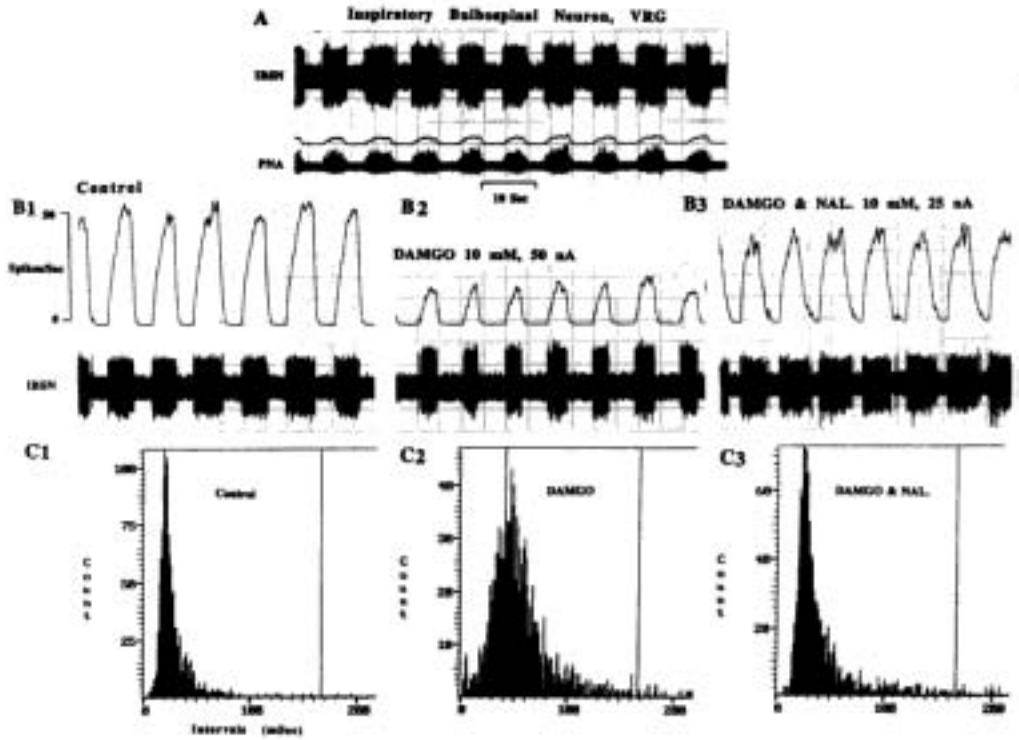

Fig. 3. Inhibition of action potential discharge frequency and burst duration in a medullary bulbospinal inspiratory neuron (IBSN), as revealed by ratemeter records and interspike interval histograms during microiontophoresis of DAMGO, a μ-opioid receptor agonist. A: Records from top to bottom are: action potentials recorded extracellularly with a 5-barrel microiontophoresis assembly, ratemeter records (moving averages) of a population of inspiratory motor axons of the phrenic nerve innervating the diaphragm and the electroneurograms of phrenic discharge activity (PNA). B: Ratemeter records (moving averages) of IBSN action potential frequency and microelectrode recordings. C: Interspike interval histograms of IBSN discharge.

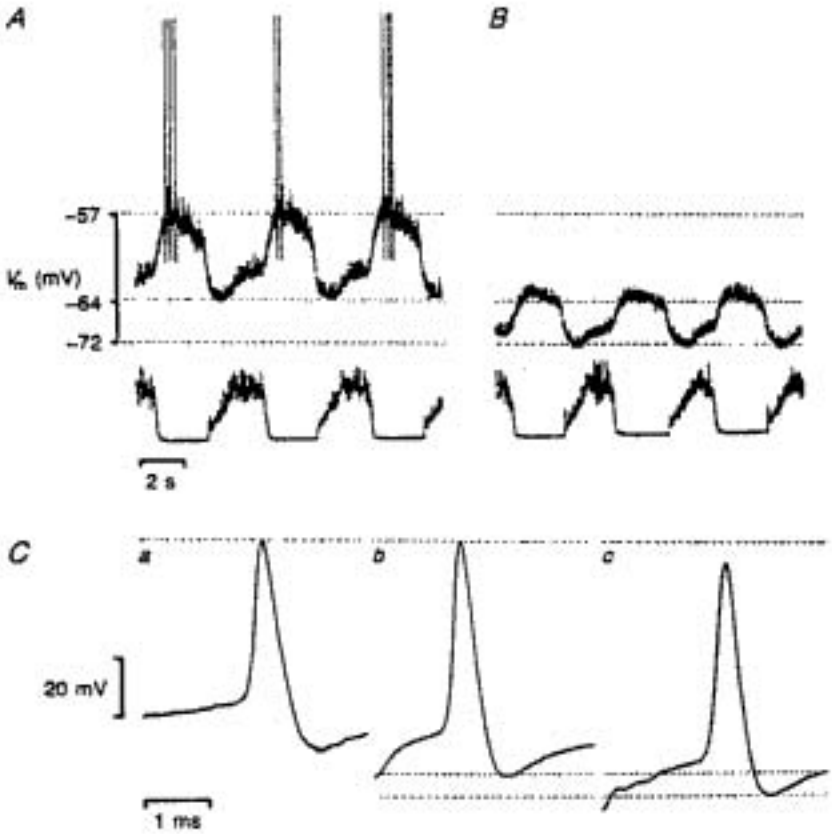

Fig. 4. Depression of cell discharge, hyperpolarization of membrane potential and decreased neuronal input resistance produced during microiontophoresis of the 5-HT-1A receptor agonist 8-OHDPAT. **A** and **B** show membrane potential (*top*; V_m) of a medullary expiratory neuron and rate-meter records (moving average) of phrenic nerve activity (*bottom*). **A**: Control records. **B**: 8-OH-DPAT microiontophoresis hyperpolarized membrane potential and abolished action potentials. Ca: Averages of 20 spontaneously occurring action potentials recorded under control conditions. Cb: Average of 20 action potentials evoked by depolarizing constant current pulses recorded in balanced bridge mode in the absence of 8-OHDPAT application. Cc: Average of action potentials evoked in the presence of 8-OHDPAT. Membrane potential is hyperpolarized, amplitude of depolarizing electrotonic potential is decreased, signifying increased membrane conductance resulting from postsynaptic inhibition.

cate whether the effects of an iontophoresed neurochemical are direct on the target neuron, or affected through actions on a presynaptic neuron. Figure4 from Lalley et al. (1994) illustrates how R_N measurements revealed that inhibition mediated by 8-OHDPAT, a 5HT-1A receptor agonist, was direct on a medullary respiratory neuron.

A mechanism of postsynaptic inhibition is consistent with data showing that 8-OHDPAT hyperpolarizes the vast majority of CNS neurons having 5-HT-1A receptors, and does so in association with increased membrane permeability to potassium ions (Zifa and Fillion 1992). Had the depression of neuron discharges been associated with reduced waves of depolarization and increased input resistance, presynaptic inhibition would have been implicated.

Dose-Response Relationships

In studies of classical dose-response relationships, the concentrations of drugs are known and it is assumed that drugs are uniformly distributed around a finite number of receptors, such that the response will vary in proportion to the number of agonist-

activated receptors. Such conditions may exist *in vitro* when drugs in a bath solution are applied to thin tissue slices, hence sigmoidal dose-response curves can be constructed for analyzing drug-receptor interactions (Gero 1971). In microiontophoresis experiments, the quantity of drug around the target neuron is generally unknown. This is because: (1) The amount of drug released from the micropipette cannot be calculated from the ejecting current (2); it is not known how the concentration of drug at the site of action is related to the distance from its release site.

Amount of Drug Released by Current

Microiontophoresis puts into action Faraday's laws of electrolysis, which state that the mass of a charged substance produced at an electrode in an aqueous solution by electrolysis is directly proportional to the quantity of electricity that has passed through the solution and the equivalent weight of the substance. Mathematically, Faraday's laws can be expressed as:

$$M = n(IT / ZF) \quad (1)$$

where M is the number of moles of the charged substance, I is the applied current (amperes), T is the duration of current application (seconds), Z is the valence state of the substance, F is Faraday's constant and n is the transport number, a proportionality factor which depends on the chemical properties of the charged substance and solvent medium.

In microelectrophoresis experiments, M is the number of moles of substance expelled at the micropipette tip by current. This means that a current applied to a wire inserted in a micropipette will repel charged molecules of similar polarity from the micropipette solution into the surrounding liquid medium, and retain molecules if the current and charge on the molecule are of opposite polarity. For example, positive current passed through a micropipette solution containing acetylcholine chloride (ACh^+Cl^-) in distilled water will expel ACh^+ and retain Cl^-. Negative current will retain ACh^+ and expel Cl^-. Substances are mobilized in aqueous solution not only by electrolysis, but also by electro-osmosis, in which the charge mobilizes the substance by acting on molecules of water of hydration that surround it. According to Curtis (1964), the contribution of electro-osmosis to the total ejection of highly charged molecules is relatively small, whereas it is the principal means of expelling poorly-charged substances. For example, hydrochloride, methochloride or methobromide salts of bicuculline, a convulsant alkaloid which blocks $GABA_A$ receptors, are dissolved in a solution of 165 mM NaCl, acidified to pH 3 and ejected by electro-osmosis with positive current.

The movement of a substance in solution is affected by the tip potential (TP) and by the zeta potential, ζ, an electrokinetic potential set up at the glass-solution interface in the interior of the micropipette tip. The total TP, to which ζ is contributory, is normally negative in sign. TP is higher when micropipette tips are finer (electrode resistance is high) and the solutions inside and outside the micropipette are dissimilar, and lower when the filling solutions are acidic.

Critical to determining the quantity M of a neuroactive substance expelled by a micropipette is an accurate determination of n. The transport number is influenced by temperature, ζ, electrode tip diameter, composition of the pipette class, the solvent, the various molecules within the pipette solution and the properties of the medium into which substances are iontophoresed. The transport number of a substance varies significantly between micropipettes even when glass composition is identical, tip sizes are similar and the test substance is expelled into identical solutions such as Ringer's solution. Values of **n** for relatively few chemicals have been measured by ejecting radioactive

compounds into distilled water, Ringer's or physiological saline solutions; however, there are large differences between values of **n** obtained in such solutions and in brain tissue (Hoffer et al. 1971). Other factors that affect n are:

- Changes in the charge at micropipette tips resulting from passage of current
- Migration of chemicals away from the tip during application of retaining currents
- Current transfer between adjacent micropipettes

A further complication is that n will change during testing if the chemical properties of nervous tissue are altered, as might occur from multiple tissue penetrations, edema, changes of pH, etc.

Concentration-Distance Relationship

Estimates have been made of the concentration of a drug at a target neuron by applying concentration-distance equations developed from the principles of diffusion. Nicholson and Phillips (1981) evaluated the effect of distance and duration of iontophoretic application on drug concentration in the vicinity of cerebellar Purkinje neurons from the equation:

$$C(d,t) = (Q\lambda^2 / 4\pi D\alpha d) \times \mathrm{erfc}(d\lambda / 2\sqrt{A}(Dt)) \quad (2)$$

where C is the concentration of the drug at a distance d, and at time t of drug application, Q is the amount of drug expelled at microelectrode tip (mols/s), λ is the tortuosity factor in the medium from source to target, α is the volume fraction, erfc is the error function and D is the diffusion constant ($cm^{-2}s^{-1}$). In the experiments of Nicholson and Phillips the test substances were measured directly in the tissue, so that C, d and t were known and α, λ could be estimated. However, in most studies, measurements of C are not possible or practical. Perhaps the only term in equation (2) which can normally be determined with certainty is **d** when compound electrodes are used to record responses from impaled neurons. Otherwise it is doubtful that accurate estimation of the actual concentration can be made, for several reasons.

First, in equation (2), Q varies with transport number and ejecting current:

$$Q = nI / F \quad (3)$$

and values of n, for reasons presented above, are not trustworthy.

Second, λ and α will vary in different regions of the nervous system and with experimental conditions.

Third, D will be influenced by:

- Enzymatic breakdown, neuronal and glial uptake of microiontophoresed chemicals.
- Barriers created by neurites, glial cells, blood vessels and tissue debris resulting from microelectrode insertion. Such barriers can block access to a neuron or cause non-uniform distribution of the ejected substance.

Estimating Relative Potency

Since concentrations are not usually measured directly and cannot be estimated with accuracy, an alternative approach is to measure the relative potencies of drugs. For accurate estimates of relative potency, the following prerequisites must be satisfied:

- Comparisons must be restricted to neurons of the same functional type, having similar electrophysiological and morphological (size, dendritic arborization) properties. Test substances must be compared for relative effectiveness on the same neuron.

- Test substances must be ejected and responses must be compared under identical recording conditions.
- At each current intensity and for each substance, the duration of drug ejection must be long enough to achieve a steady response level.
- Recovery to control levels must be allowed between drug ejections.
- Responses should be normalized for the analysis of dose-response properties.

Two examples are given here to indicate how relative potencies of drugs are determined and antagonist efficacy is evaluated.

Example 1

Curtis et al. (1971) analyzed the dose-related inhibitory effects of the amino acid glycine and the effectiveness of strychnine as an antagonist on spinal interneurons in the following manner:

- A steady level of cell discharge was evoked with microiontophoretic application of DL-homocysteic acid, an excitatory glutamate-like amino acid.
- Glycine was applied during the steady state discharge with different ejecting currents to effect graded degrees of depression of spike frequency.
- The sequence was repeated two more times during which strychnine was applied with 5 nA and 10 nA ejecting currents. Glycine and strychnine were applied sufficiently long to obtain steady levels of response.
- Results were plotted graphically as percent of maximum inhibition of discharge frequency (ordinate) vs. logarithm of glycine-ejecting current (nA). As shown in Figure 5A, the curves thus derived were sigmoidal. Strychnine produced current-related shifts to the right, consistent with competitive antagonism at glycine receptors. Curtis et al. (1971) pointed out that difficulties associated with such studies of dose-response relationships can arise by virtue of non-uniform drug distribution, although such problems were not encountered in their study.

Example 2

Hill and Simmonds (1973) and Simmonds (1974) used similar microiontophoresis procedures, but utilized a different method of graphical analysis. In their investigation:

- Steady firing of cortical neurons was evoked with glutamate iontophoresis.
- Graded levels of discharge depression were evoked by GABA applied with different ejecting currents.
- As shown in Figure 5B, plots of % inhibition of firing vs. log of ejection time yielded sigmoidal curves which shifted in parallel with different intensities of ejecting currents. The index of relative potency was chosen as T50, the time taken to achieve 50 % inhibition of neuronal firing (indicated by the dashed horizontal line).

Time-response curves for GABA were also displaced to the right along the time axis by microiontophoretic application of $GABA_A$ receptor antagonists. The displaced curves were parallel to the control curve when responses were plotted against linear time rather than log time. Relative effectiveness of antagonists was assessed by comparing values of T50.

Critical to the accuracy of such procedures is the requirement of restricting comparisons to neurons of similar type. This eliminates dissimilarities in responsiveness which are linked to different intrinsic drug sensitivities among different types of neurons.

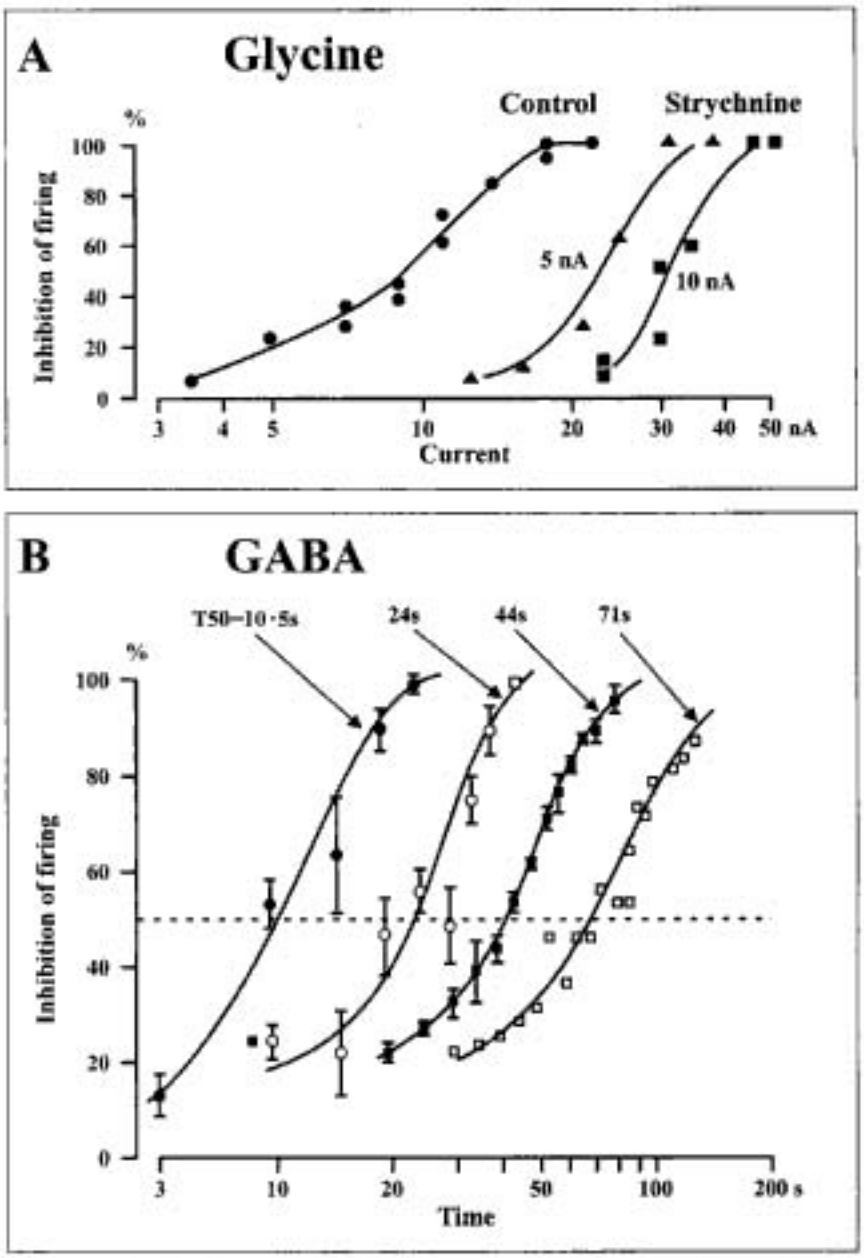

Fig. 5. Graphical analysis of the relative potencies of receptor agonists and antagonism by a competitive receptor antagonist applied by microiontophoresis. **A:** Plots of inhibition of action potential frequency in cat spinal interneurons by glycine and antagonism by strychnine. Glycine was tested on a steady level of action potential firing produced by iontophoresis of DL-homocysteic acid. Ordinate, percent of maximal inhibition of action potential frequency by different glycine-ejecting currents. Abscissa, logarithm of ejecting current. Control curve (glycine only) is on the *left*. *Middle*, concurrent ejection of glycine and strychnine, 5nA. *Right*, ejection of glycine and strychnine, 10nA. Note parallel shifts produced by strychnine, indicative of competitive antagonism at glycine receptors. (Modified from Curtis et al., 1971). **B:** Plots of inhibition of cat cortical neuron discharges during microiontophoresis of γ-aminobutyric acid (GABA) with four different currents: 20nA (•), 10 nA (o), 5nA (■) and 2nA (□). Steady firing of the neuron was induced by microiontophoresis of L-glutamate, 20nA. Vertical bars represent standard errors. Values of T50 are times required to produce 50% inhibition of evoked firing by GABA. (Reproduced from Hill and Simmonds 1973).

Potential Sources of Error

Problems of reproducibility and interpretation will arise if sources of artifact are not eliminated. Those associated with pH and current effects have already been discussed. Other potential sources of error include undesired local anesthetic and other types of non-selective drug actions, network-related changes in cellular behavior and presynaptic effects.

- Local Anesthetic and Other Non-Selective Drug Actions
  Certain drugs including muscarinic-, adrenergic- and serotonin-receptor blocking drugs have local anesthetic effects at concentrations greater than those responsible for their primary effects. The hallmark of local anesthetic activity is depression of action potential amplitude and lengthening of duration. At high concentrations, drugs may also non-selectively depress spike discharges. Such effects can usually be eliminated by reducing the strength of ejecting currents.
  Other examples of non-selective effects include enhancement of responses to depressant substances such as GABA by barbiturates and other general anesthetics (Nicoll et al. 1990), and blockade of receptors for other neurotransmitters by "selective" receptor antagonists, for example blockade of β–adrenoceptors by Ketanserine, a 5-HT-2A receptor blocker. In the former situation, it may be necessary to perform tests on unanesthetized decerebrate preparations. In situations such as the latter, it is important that the test substance be not only selective for a subtype of receptor, but also be free of actions on receptors for other neurotransmitters, neuromodulators and hormones.
- Changes Related to Altered Network Properties
  Changes in the responsiveness of neurons to microiontophoresed substances may occur during the course of testing because the intensity of spontaneous synaptic input to neurons changes. Synaptic input may vary with anesthetic state, blood pressure, alveolar and arterial oxygen and carbon dioxide, etc. It is therefore imperative to monitor and minimize variables that are known to affect the network behavior of test neurons. It is also helpful to record some index of network behavior, such as discharges of a phrenic nerve when testing brain stem or spinal respiratory neurons, or blood pressure when testing sympathetic vasomotor neurons.
- Presynaptic Effects
  Drug actions on presynaptic neurons should be suspected if effects on nerve cell properties are opposite to the known actions of the drug. For example, presynaptic depression would be suggested if cell discharges increase during application of a depressant drug. Presynaptic actions should also be suspected if receptor agonists and antagonists have similar effects on the target neuron (Wang et al. 1995).

Cell Structure and Interpreting Functional Significance

Responsiveness to a neuroactive substance will be influenced by the location of receptors. Hence, cell size and geometry should be taken into account when interpreting the functional significance of endogenous neurochemicals based on responses to iontophoresis. For example:

- Small cells are usually more sensitive than large cells to microiontophoretically applied drugs because, for the same dose, a relatively greater percentage of the cell surface comes into contact with the drug. The greater sensitivity might lead to an erroneous conclusion that excitability in small cells is more effectively modulated under physiological circumstances by an endogenous neurohumoral substance.

- Greater numbers of receptors for neurotransmitters and neuromodulators may be located at distant dendritic synapses. A weak or absent response to iontophoresis may mean that the neurochemical has not reached remote synapses where its endogenous counterpart has physiologically important actions.

Subprotocol 2
Micropressure Ejection

General Uses and Description

Pressure ejection is used to deliver uncharged or poorly charged substances to the vicinity of neurons. The technique is useful for *in vivo* and *in vitro* studies. In the *in vitro* slice preparation, separate recording and pressure-ejection pipettes are positioned close to the target neuron. In the *in situ* preparation, micropressure ejection has been used in two general ways:

1. Single micropipettes with relatively large tips (10µm or greater) have been used to inject nanoliter volumes for the purpose of altering the excitability of groups of neurons in a small area. For example, nanoliter volumes of solutions containing GABA- and glycine receptor antagonists have been ejected into the Pre-Boetzinger Complex of the medulla oblongata, a critical respiratory rhythm-generating area, to evaluate the significance of inhibitory synaptic transmission on the respiratory rhythm in the adult mammal *in vivo* (Pierrefiche et al., in press, 1998).
2. Volumes less than 1 nl of neuroactive drugs and neuromodulators have been applied to single neurons from multibarrel assemblies during extracellular or intracellular recording (Palmer et al. 1986; Szente et al. 1990).

Volumes of neuroactive substances are ejected from micropipettes connected by soft catheter tubing and high-pressure tubing to a source of compressed gas, usually nitrogen. A switch- or TTL pulse-controlled solonoid valve is used to deliver pulses of desired pressure and duration, either as single pulses are as programmed pulses.

Materials

Equipment and Supplies

Equipment for electrophysiological recording and analysis is the same as described in Chapter 5 and in Part 1 of this chapter. Additional equipment and supplies include:

- A source of inert gas, such as a cylinder of pressurized nitrogen.
- Electronically-controlled solonoid pressure valves to regulate degree and duration of pressure
- A source of trigger pulses for the pressure valves (TTL pulse generator)
- Surgical microscope equipped with reticule to measure displacement of solutions from micropipettes
- High-pressure tubing and soft catheter tubing to connect pipettes to output ports of pressure valve.

Capillary Glass

Single or multibarrel pipettes containing filaments are used to aid filling. Multibarrel assemblies are commercially available. In most cases the barrels are fused all along the length of the assembly, hence it is not possible to attach tubing to each barrel to independently apply various substances. However, assemblies can be fabricated as described

in above. Procedures for assembling multi-barrel pipette assemblies are also described by Palmer et al. (1986).

Solutions

Drugs should be dissolved in mock CSF or Ringer's solution, preferably in low micromolar concentrations and adjusted to pH 7.4.

Procedure

Test procedures are the same as those described for microiontophoresis. The performance of the pressure-ejecting system should be checked before insertion of the assembly in tissue. Connections between micropipette and catheter tubing (3–5 inch lengths) should be secure. Once in the tissue, performance of the pressure system should be checked again. Drug volumes ejected with various pressures and pulse durations should be selected and preliminary tests of drug effectiveness on non-target neurons should be made whenever feasible.

Methods of Analysis

Methods of analysis are identical to those described for microiontophoresis. The procedures described by Curtis et al (1971) and Hill and Simmonds (1973) are very suitable for comparing relative potencies of drugs and the effectiveness of antagonists.

Calculating Volume and Quantity Ejected

The general procedure is to microscopically measure with a reticule the length (L) of fluid ejected under pressure from a pipette of known internal radius (r). Volume (V) can then be calculated from $V = \pi r^2 L$. From the known concentration of the pipette solution, the amount of substance ejected can be calculated; however, the concentration of drug at the target site will not be known (see above: Concentration-Distance Relationships).

Volume Ejected vs. Micropipette Resistance

Micropipettes with tip resistances between 1.0 and 1.4 MΩ resistance will usually eject uniform volumes for a given pressure and duration over long test periods. Pipettes with finer tips tend to plug in brain or spinal cord tissue whereas larger tips (resistance less than 1.0 MΩ) produce variable results, and larger volumes are ejected that are more likely to produce volume-related response artifacts (Sakai et al. 1979; Palmer et al. 1986).

Artifacts

pH, Local Anesthetic and Other Nonspecific Effects

Response artifacts similar to those that might be encountered with microiontophoretic application can be produced by micropressure ejection. It is therefore important to:

- Adjust pH as close as possible to pH 7.4. Solutions of pH less than 5.5 and greater than 8 should not be used.
- Drug concentrations in pipette solutions should be dilute, preferably in the low micromolar range.

Volume Artifacts

Solution volume can produce injury discharges and abrupt changes of membrane potential. Larger volumes can also move cells away from the recording micropipette. Such artifacts should be suspected when changes of neuron discharge, membrane potential, or neuronal network behavior occur simultaneously with the volume ejection. Artifacts can be tested for by ejecting drug-free solution from a barrel of the assembly.

Solvent Artifacts

Solvents other than water, such as ethanol or dimethylsulfoxide, may alter neuron behavior. Hence, they should be diluted as much as possible with Ringer's solution or

mock CSF. Solutions of high osmolarity should also be avoided, since they might affect neuron responses through redistribution of cytoplasmic and extracellular water (Curtis 1964).

Comments

The methods described in this chapter have turned out to be very useful for delivering drugs and chemicals directly to the vicinity of target neurons. Studies using these methods have provided important information about the identity and functional characteristics of neurotransmitters and neuromodulators. Cellular mechanisms of drug action have been elucidated, and signal transduction processes that link membrane receptor activation to ion channel functions have been uncovered. Methods for local drug application will continue in the future to be very useful for evaluating the direct effects of chemicals on neurons in the CNS. Modern techniques in molecular biology and genetics have led to the discovery of a large number of potentially important neuromodulators whose functional significance and intracellular transduction mechanisms await identification through the use of microiontophoresis and micropressure-ejection.

Suppliers

Programmable Multi-Channel Microintophoresis Pumps

Micro-Iontophoresis Current Generator 6400	Dagan Corporation, USA
MVCS Iontophoretic Series	npi electronic GMBH, FRG
Neurophore BH-2	Medical Systems Corp, USA

Electronically-controlled Pressure Valve Systems

Picospritzer	General Valve Corporation, USA
PPM-2	Medical Systems, USA
Pneumatic Picopump PV830	World Precision Instruments, USA
Hydraulic NanoPump, A1400	World Precision Instruments, USA
Nanoliter Injector A203	World Precision Instruments, USA
PDES-2/4	npi elecronic GMBH, FRG

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